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Identification and Measurement of Endogenous β-Oxidation Metabolites of 8-*epi*-Prostaglandin F2α*

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F_2-isoprostanes are prostaglandin-like compounds derived from nonenzymatic free radical-catalyzed peroxidation of arachidonic acid. 8-*epi*-Prostaglandin (PG) $F_{2\alpha}$, a major component of the F_2-isoprostane family, can be conveniently measured in urine to assess noninvasively lipid peroxidation *in vivo*. Measurement of major metabolites of endogenous 8-*epi*-$PGF_{2\alpha}$, in addition to the parent compound, may be useful to better define its formation *in vivo*. 2,3-Dinor-5,6-dihydro-8-*epi*-$PGF_{2\alpha}$ is the only identified metabolite of 8-*epi*-$PGF_{2\alpha}$ in man, but its endogenous levels are unknown. In addition to this metabolite, we have identified another major endogenous metabolite, 2,3-dinor-8-*epi*-$PGF_{2\alpha}$, in human and rat urine. The identity of these compounds, present at the pg/ml level in urine, was proven by a number of complementary approaches, based on: (*a*) immunoaffinity chromatography for selective extraction; (*b*) gas chromatography-mass spectrometry for structural analysis; (*c*) *in vitro* metabolism in isolated rat hepatocytes; and (*d*) chemical synthesis of the enantiomer of 2,3-dinor-5,6-dihydro-8-*epi*-$PGF_{2\alpha}$ as a reference standard. In humans, the urinary excretion rate of both dinor metabolites is comparable with that of 8-*epi*-$PGF_{2\alpha}$. Both metabolites increase in parallel with the parent compound in cigarette smokers, and they are not reduced during cyclooxygenase inhibition. Another β-oxidation product, 2,3,4,5-tetranor-8-*epi*-$PGF_{2\alpha}$, was identified as a major product of rat hepatocyte metabolism. In conclusion, at least two major β-oxidation products of 8-*epi*-$PGF_{2\alpha}$ are present in urine, which may be considered as additional analytical targets to evaluate 8-*epi*-$PGF_{2\alpha}$ formation and degradation *in vivo*.

F_2-isoprostanes are a complex family of isomeric F_2-prostaglandin (PG)-like[1] compounds derived from free radical-catalyzed nonenzymatic peroxidation of arachidonic acid (1). These compounds are currently used as novel biomarkers of lipid peroxidation *in vivo* (2–4). F_2-isoprostanes esterified to tissue or plasma lipids or in the free form in body fluids have in fact proved to be useful indices of local or systemic oxidant stress (2–4). Assay of selected major isomers such as 8-*epi*-$PGF_{2\alpha}$ or isoprostane $F_{2\alpha}$-I in human urine has proven an accurate noninvasive means of evaluating their formation *in vivo* (3, 5).

In view of further refining the quantitative assessment of the endogenous formation of F_2-isoprostanes, it would be important to investigate their degradation *in vivo*. The identification of the major metabolites of selected F_2-isoprostanes would be useful for two main reasons. First, quantitation of major metabolites in addition to the parent compound may allow a more accurate evaluation of the overall production of the biomarker *in vivo* while adding significance to individual measurements. Second, identification of F_2-isoprostane metabolites may help finding circulating compounds that can be measured without the risk of artifactual production *ex vivo* (2, 4).

Theoretically, metabolism of F_2-isoprostanes may proceed as for F_2-prostaglandins (6–8), *i.e.* they may undergo various combinations of β-oxidation, double bond reduction, alcohol group oxidation, ω-hydroxylation, and ω-oxidation, which *per se* may lead to a miriad of metabolites. The structural differences between the four regioisomers and the stereochemical differences within each regioisomer class (1) will likely cause major differences in the way each product is degraded, further complicating the picture. Therefore, metabolism of F_2-isoprostanes cannot be studied with a single parent compound as a model nor can a single type of metabolite be chosen to monitor degradation of F_2-isoprostanes as a group. Moreover, administration of relevant amounts of a single isomer to study its catabolism may somewhat alter its metabolic pathway, possibly favoring reactions that are normally inhibited by the presence of other competing endogenous substrates. Therefore, a reasonable approach to characterize F_2-isoprostane catabolism would be to identify the major endogenous metabolites of the principal isomers produced *in vivo*.

To date, two distinct F_2-isoprostane isomers, 8-*epi*-$PGF_{2\alpha}$ and isoprostane $F_{2\alpha}$-I (5, 9), have been positively identified *in vivo*. Metabolism of [^{3}H]8-*epi*-$PGF_{2\alpha}$ has been studied by Roberts *et al.* (10) in humans, and a single compound, 2,3-dinor-5,6-dihydro-8-*epi*-$PGF_{2\alpha}$, has been identified as the major urinary metabolite. The same group identified other F_2-isoprostane metabolites as a group of C16 dioic compounds of unknown stereochemistry, with two hydroxyl groups, one keto group, and one double bond (11). We report here the identification of two endogenous urinary metabolites of 8-*epi*-$PGF_{2\alpha}$ by a strategy taking advantage of immobilized antibodies to capture endogenous cross-reactants for subsequent identification/measurement by gas chromatography-mass spectrometry (12, 13).

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[1] The abbreviations used are: PG, prostaglandin; IAC, immunoaffinity chromatography; PFB, pentafluorobenzyl; TMS, trimethylsilyl; tBDMS, *tert*-butyldimethylsilyl; BB, butyl boronate; ME, methyl ester; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; EI, electron impact; SIR, selected ion monitoring; SPE, solid phase extraction.

EXPERIMENTAL PROCEDURES

Materials—8-*epi*-PGF$_{2\alpha}$ and 3,3′,4,4′-[$^2H_4$]-8-*epi*-PGF$_{2\alpha}$ were purchased from Cayman Chemicals (Ann Arbor, MI). PGF$_{2\alpha}$ and 3,3′,4,4′-[$^2H_4$]-PGF$_{2\alpha}$ were from Upjohn Co. (Kalamazoo, MI). *ent*-12-*epi*-PGF$_{2\alpha}$ (15*S* and 15*R*), 12-*epi*-PGF$_{2\alpha}$ (15*S* and 15*R*), and 15*R*-8-*epi*-PGF$_{2\alpha}$, were synthesized in our laboratory as described previously (14, 15).

Immunoaffinity Chromatography—IAC extractions were performed as described previously, using immunosorbents prepared with antibodies raised against 8-*epi*-PGF$_{2\alpha}$ (16). Briefly, urine was diluted (1:2–1:5) with 50 mM phosphate buffer (pH 7.4) spiked with $^2H_4$-labeled internal standard, if required, and percolated slowly through a IAC column prepared with the immunosorbent (IgG fraction of the antiserum coupled to CNBr-activated Sepharose 4B). After washing with distilled water, the column was eluted with acetone:water (95:5, v/v), and the eluate was dried under an air stream.

Derivatization—Samples were converted to various derivatives before GC-MS analysis. Pentafluorobenzyl (PFB) ester and trimethylsilyl (TMS) ethers were prepared as described (14), *tert*-butyldimethylsilyl (tBDMS) ethers by treating dried samples with 10 μl of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA, Pierce, Rockford, IL) plus dry pyridine (10 μl) and acetonitrile (30 μl) for 1 h at 60 °C, cyclic butyl boronate derivatives (BB) by adding 1-butane boronic acid (60 μl of a 0.3 mg/ml solution in acetonitrile) for 10 min at 40 °C, and methyl esters (ME) by addition of ethereal diazomethane (150 μl) at room temperature.

GC-MS—GC-negative ion chemical ionization (NICI)-MS was performed as described (16) using a Finnigan 4000 mass spectrometer. Briefly, GC operating conditions were: NB-54 (5% phenyl, 95% dimethylpolysiloxane) or CP-Sil 19 CB (14% cyanopropylphenyl, 86% dimethylpolysiloxane) fused silica capillary columns (length, 25 m; inner diameter, 0.32 mm; film thickness, 0.12 μm); solvent-split injection (50 to 300 °C); oven temperature, isothermal at 120 °C for 1 min, and then programmed to 300 °C at 25°/min; helium was used as a carrier gas. NICI operating conditions were: selected ion recording (SIR) of carboxylate anions (M-181, loss of $^{\cdot}CH_2C_6F_5$); full scan mode: 100–850 *m/z*; methane as reagent gas; electron energy, 100 eV. GC-electron impact (EI)-MS was performed using a VARIAN SATURN 2000 Ion Trap mass spectrometer. GC operating conditions were: HP-5 MS (5% phenyl, 95% dimethylpolysiloxane) fused-silica capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm); splitless injection (240 °C); oven temperature, isothermal at 160 °C for 1 min, and then programmed to 310 °C at 20°/min; helium as carrier gas. MS conditions were: ion trap temperature, 200 °C; mass range, 50–650 *m/z*; electron energy, 70 eV.

Metabolite Quantitation by IAC-GC-NICI-MS—To control IAC extraction efficiency for metabolite quantitation without stable isotope-labeled analogues, we devised the following procedure: (*a*) test urine samples containing different pre-determined amounts of endogenous 8-*epi*-PGF$_{2\alpha}$ were percolated in succession through three IAC columns *without* internal standard; (*b*) each column was then eluted separately in tubes containing a fixed amount of the internal standard $^2H_4$-8-*epi*-PGF$_{2\alpha}$; (*c*) GC-NICI-MS quantitation of the metabolites recovered in the three IAC eluates from the same urine sample showed that all compounds had been extracted almost completely in the *first two rounds* (96–100% for 8-*epi*-PGF$_{2\alpha}$ and 2,3-dinor-8-*epi*-PGF$_{2\alpha}$, 87–92% for 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$). Two-round extractions were therefore performed for unknown samples, the total amount of each metabolite being calculated by summing the two partial results obtained. Quantitation was done by using standard plots of 8-*epi*-PGF$_{2\alpha}$/$^2H_4$-8-*epi*-PGF$_{2\alpha}$ peak area ratio (*y*) *versus* the amount of 8-*epi*-PGF$_{2\alpha}$ (*x*) (r = 0.9999), assuming that equimolar amounts of the C20 and C18 analogues give similar carboxylate anion peak area response, because this ion carries most of the total ion current.

Chemical Synthesis of ent-2,3-Dinor-5,6-dihydro-8-epi-PGF$_{2\alpha}$—The total synthesis of the enantiomer of (15*S*)-2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ was carried out as we have described recently (17) using diacetone-D-glucose as starting material. After methylation of the carboxyl acid with diazomethane, the 15*R*-epimer (*ent*-2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$) could be separated from the 15*S*-epimer by flash chromatography over silical gel. Relative stereochemical assignments were made by NOE ^1H NMR study.

C Values—A mixture of normal saturated C18-C24 fatty acids were derivatized to PFB esters and analyzed by GC-NICI-MS in the SIR mode, monitoring their carboxylate anions (M-PFB). Plots of retention times (*y*) with two different GC columns against carbon number (*x*) (r = 0.999) were used to calculate equivalent *C* values from the retention times of different PFB derivatives (TMS, BB-TMS, or tBDMS) of each metabolite (Table I). The same procedure was used to calculate *C* values of ME-TMS metabolites with C18-C24 fatty acid methyl esters (see "Results").

Human Urine—6-h urine was collected from healthy volunteers (8 males, 2 females; age, 24–57 years; 5 smokers and 5 nonsmokers). Four nonsmokers were given two oral doses of naproxen sodium (550 mg at 12-h interval), and urine was collected before treatment and after the second dose. Samples were stored at −20 °C.

Rat Urine—24-h urine was collected from male Crl:CD (SD)BR rats (300–350 g) kept in metabolic cages. 8-*epi*-PGF$_{2\alpha}$ (10 μg dissolved in 0.5 ml of sterile phosphate-buffered saline) was injected intravenously into a rat whose urine was collected for 24 h before and after treatment. Samples were kept at −20 °C until analyzed. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national[2] and international laws and policies.[3]

Isolated Rat Hepatocytes—Hepatocytes were isolated from fed male Crl:CD (SD)BR rats (230–260 g) by perfusing the liver with a collagenase solution (collagenase type IV, Sigma) by the method of Seglen (18). After removing the liver capsule, the cell suspension was passed through gauze. The hepatocytes were washed three times (50 *g* × 1 min) and suspended in Leibovitz L-15 medium (Life Technologies, Inc., Paisley, Scotland) supplemented with 18 mM Hepes, 1 μg/ml insulin, 50 μg/ml gentamicin, and 5% fetal calf serum (Life Technologies, Inc.) at a cell density of 1 × 10^6 cells/ml. The viability, assessed by trypan blue exclusion, was higher than 85%. After 3 h of adhesion the medium was changed with an equal medium without fetal calf serum and gentamicin, and the cells were incubated for 20 min with and without 8-*epi*-PGF$_{2\alpha}$ or PGF$_{2\alpha}$. The cell-free incubation medium was collected and stored at −20 °C until analyzed. Metabolites were extracted both by IAC and by C18 solid phase extraction (SPE). The latter was performed by loading the sample at pH 3.5, washing with water and petroleum ether, and then eluting with methyl formate. Aliquots of IAC and C18 SPE extracts were dried and derivatized to ME-TMS or PFB-TMS and analyzed by GC-EI-MS or GC-NICI-MS, respectively.

RESULTS

Search Strategy for β-Oxidation Metabolites of Endogenous 8-epi-PGF$_{2\alpha}$—Because polyclonal antibodies raised against prostanoids coupled to the carrier protein via the carboxyl group often cross-react with the corresponding β-oxidation products (12, 13, 19), we tested three antibodies (A, B, and C) raised against 8-*epi*-PGF$_{2\alpha}$ (16) for their ability to extract any of its possible α-chain-shortened (C18 and C16) metabolites. Aliquots (10 ml) of a human urine pool were extracted using three different immunosorbents prepared with A, B, and C antibodies. Extracts were derivatized as PFB-TMS and first analyzed by GC-NICI-MS in the SIR mode, monitoring the carboxylate anions of 8-*epi*-PGF$_{2\alpha}$ (*m/z* 569) and those of its putative C18 and C16 homologues (*m/z* 541 for C18:2, *m/z* 543 for C18:1, and *m/z* 515 for C16:1). The three immunosorbents showed different selectivity toward the C18 and C16 metabolites, with A extracting only 8-*epi*-PGF$_{2\alpha}$ and no metabolites and B extracting 8-*epi*-PGF$_{2\alpha}$ and a single C18:2 metabolite (I), whereas C extracted 8-*epi*-PGF$_{2\alpha}$ plus PGF$_{2\alpha}$ and doublets of their chain-shortened homologues C18:2 (I and Ia) and C18:1 (II and IIa) as well as a single C16 metabolite (IIIa) (Fig. 1). The GC behavior of these compounds preliminarily suggested that I and II were metabolites of 8-*epi*-PGF$_{2\alpha}$, whereas Ia, IIa, and IIIa were derived from PGF$_{2\alpha}$ (Table I).

Identification of Endogenous Urinary Metabolites—Structural identification of immunoextracted urinary metabolites was achieved by different complementary approaches, all suggesting that they were the following: 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ (I) and 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ (II), plus

[2] Decreto Legislativo Number 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, Gazzetta Ufficiale, 14 Luglio 1994.

[3] European Economic Community Council Directives 86/609, Official Journal L 358, 1, Dec. 12, 1987; Guide for the Care and Use for Laboratory Animals, U.S. National Research Council, 1996.

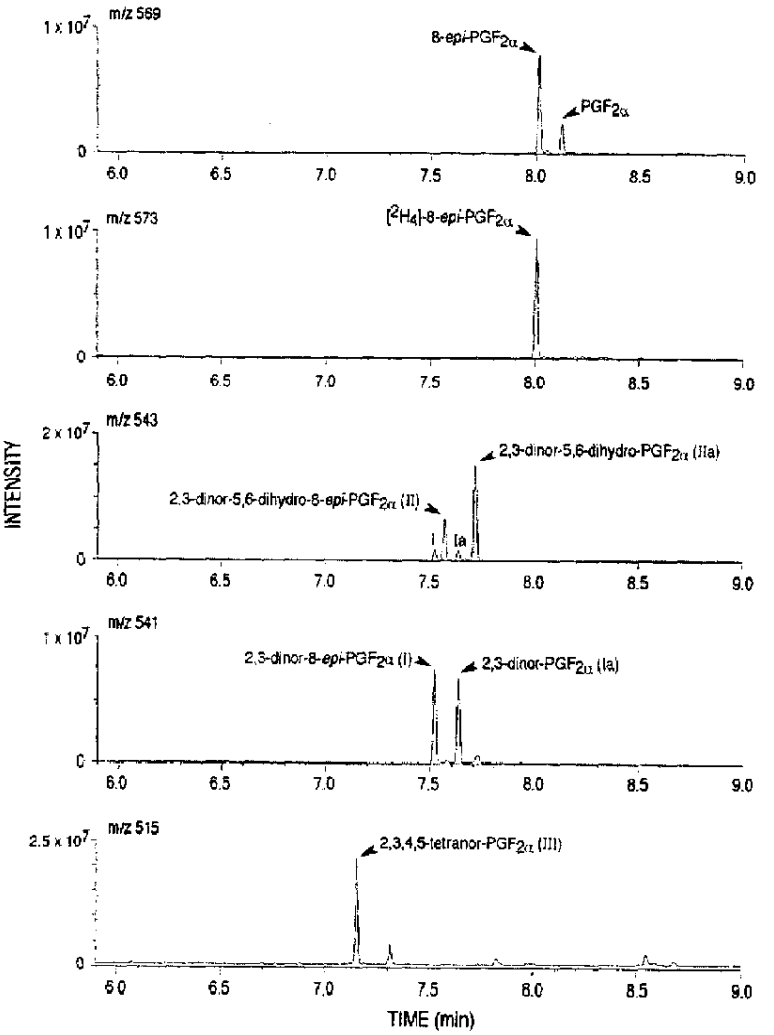

FIG. 1. **GC-NICI-MS selected ion recording tracing of human urine extracted by immunoaffinity chromatography (immunosorbent C).** The ions monitored for 8-*epi*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ and their metabolites are the carboxylate anions (M-PFB) of the PFB-TMS derivatives. Peaks labeled as I and Ia at *m/z* 543 are from the isotopic cluster of the carboxylate anions recorded at *m/z* 541 (metabolites I and Ia). The relative amounts of the compounds cannot be directly evaluated from this tracing, because their recovery is different.

TABLE I

NICI mass spectral data and equivalent C values of different PFB derivatives of 8-epi-PGF$_{2\alpha}$, PGF$_{2\alpha}$ and their metabolites

Compound	Structure	TMS			tBDMS			BB-TMS		
		m/z	C value[a]	C value[b]	*m/z*	C value[a]	C value[b]	*m/z*	C value[a]	C value[b]
8-*epi*-PGF$_{2\alpha}$	C20:2	569	22.90	22.83	695	28.44	27.73	491	25.56	26.20
I	C18:2	541	21.12	21.09	667	26.44	26.20	463	23.85	24.57
II	C18:1	543	21.34	21.20	669	26.58	26.24	465	24.14	24.78
III[c]	C16:1	515	19.48	ND[d]	641	ND	ND	437	ND	ND
PGF$_{2\alpha}$	C20:2	569	23.27	23.15	695	29.24	28.26	491	24.94	25.43
Ia	C18:2	541	21.52	21.44	667	27.06	26.74	463	23.16	23.68
IIa	C18:1	543	21.81	21.69	669	27.38	26.95	465	23.49	23.97
IIIa	C16:1	515	19.81	19.64	641	25.45	25.20	437	21.63	22.12

[a] NB-54 column.
[b] CP SIL19 column.
[c] This compound was not observed in urine but only in rat hepatocytes.
[d] ND, not done.

their PGF$_{2\alpha}$-derived counterparts 2,3-dinor-PGF$_{2\alpha}$ (Ia) and 2,3-dinor-5,6-dihydro-PGF$_{2\alpha}$ (IIa), and 2,3,4,5-tetranor-PGF$_{2\alpha}$ (IIIa).

Selected Ion Recording of Different Derivatives—Different portions of an IAC extract of human urine (40 ml on immunosorbent C) were derivatized to PFB-TMS, PFB-tBDMS, or PFB-BB-TMS and analyzed by GC-NICI-MS in the SIR mode. Carboxylate anion *m/z* values to be monitored were calculated for the different derivatives and for each of the following putative structures: trihydroxy-dinor-prostadienoic acid (C18:2), trihydroxy-dinor-prostaenoic acid (C18:1), and trihydroxy-tetranor-prostaenoic acid (C16:1). For each derivative, peaks were registered at the expected *m/z* value with *C* values changing coordinately with the authentic parent compounds on the different columns (Table I), thus confirming the proposed structures. Additional structural information

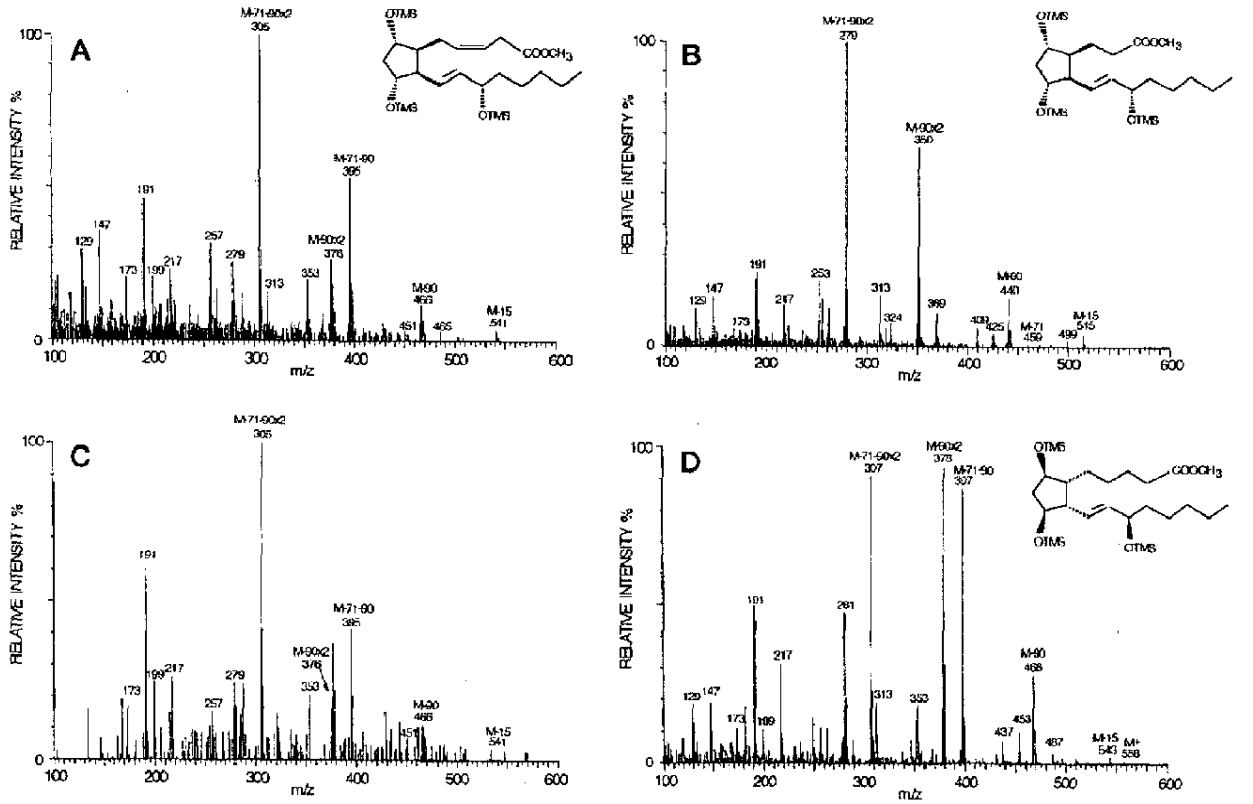

FIG. 2. **EI mass spectra of ME-TMS derivatives of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ (*panel A*) and 2,3,4,5-tetranor-8-*epi*-PGF$_{2\alpha}$ (*panel B*) from isolated rat hepatocytes incubated with 8-*epi*-PGF$_{2\alpha}$ (extracted by C18 SPE), endogenous 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ from human urine (sequentially extracted on immunosorbents C and B) (*panel C*), and chemically synthesized *ent*-2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ (*panel D*).**

was obtained by the formation of butylboronate cyclic derivatives (PFB-BB-TMS), implying that the two 9,11-hydroxyls are in the *cis* position for all compounds (11). Data regarding the C16 metabolite of 8-*epi*-PGF$_{2\alpha}$ identified in rat hepatocyte preparation (see below) are also shown in Table I. The *C* values of the C18 and C16 metabolites were about 1.5 and 3 units smaller than those of the respective parent compound for the different derivatives, as reported for two- and four-carbon α-chain shortening of F series prostaglandins (8, 20). Δ^5 double bond saturation resulted in the expected small increase in *C* value for all PFB derivatives.

Full Scan Mass Spectrometry—Full scan NICI mass spectra were obtained from a 35-ml human urine sample extracted on immunosorbent C and derivatized as PFB-TMS. The mass spectra of all metabolites revealed a carboxylate anion isotopic cluster (M-181, loss of pentafluorobenzyl group) as the only prominent peak, with the expected *m/z* values (Table I) and relative isotopic abundance (data not shown). To obtain additional evidence of the identity of urinary 2,3-dinor-8-*epi*-PGF$_{2\alpha}$, full scan EI-MS analysis was performed on a IAC extract (immunosorbent C) from a 160-ml human urine sample derivatized as ME-TMS. At the retention time of authentic *ent*-2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ (*C* value 21.69; *cf. C* value for 8-*epi*-PGF$_{2\alpha}$: 23.18), we recorded a mixed mass spectrum clearly showing doublets of major fragment ions at *m/z* 305–307, 376–378, 395–397, and 466–468, corresponding to the major fragment ions retaining the α-chain of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ (as seen in the hepatocyte preparation, Fig. 2*A* and Table II) and 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ (as seen with the authentic enantiomer, Fig. 2*D* and Table II). The difference of 2 Da in the fragment ion doublets is due to the presence or absence of the Δ^5 double bond. The two compounds coeluted when analyzed as ME-TMS in these conditions. We then prepared a similar sample but before derivatization reextracted it on immunosorbent B, thus eliminating 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$. We thus obtained a mass spectrum (Fig. 2*C*) of weak intensity but with all prominent ions corresponding to those of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ (Fig. 2*A* and Table II).

Rat Urine—Aliquots of pooled rat urine (10 ml) were also extracted on immunosorbent C and processed as described above for NICI-MS analysis. The same compounds were identified as for human urine, with the exception of the C18:2 derivative of PGF$_{2\alpha}$ (2,3-dinor-PGF$_{2\alpha}$), which was not detectable (data not shown). In a rat treated with 10 μg of intravenous 8-*epi*-PGF$_{2\alpha}$, 24-h excretion of 8-*epi*-PGF$_{2\alpha}$, 2,3-dinor-8-*epi*-PGF$_{2\alpha}$, and 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ increased by 5.6-, 9.1-, and 9.2-fold over the respective pre-treatment levels (1.16, 1.20, and 1.55 ng/24 h), suggesting that the latter compounds were metabolites of the former.

Identification of Exogenous Hepatocyte Metabolites—To confirm the identity of the urinary metabolites we verified that their gas chromatographic and mass spectral characteristics were identical to those of β-oxidation products of authentic 8-*epi*-PGF$_{2\alpha}$ or PGF$_{2\alpha}$ incubated with rat hepatocytes, which were in turn identified by EI-GC-MS as follows. Products of 8-*epi*-PGF$_{2\alpha}$ or PGF$_{2\alpha}$ metabolism by isolated rat hepatocytes were extracted in parallel by IAC (immunosorbent C) and C18 SPE. Both extracts were then analyzed by full scan EI-GC-MS. Putative 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ (Metabolite I) was present in both extracts. Its structure was proven by the mass spectrum of

TABLE II

EI-MS fragmentation pattern of ME-TMS derivatives of 8-epi-PGF$_{2\alpha}$ and its metabolites

a, α-chain:C1–C7 for 8-*epi*-PGF$_{2\alpha}$, C1–C5 for I and II, and C1–C3 for III; *b*, C9–C10 or C10–C11 for 8-*epi*-PGF$_{2\alpha}$, C7–C8 or C8–C9 for I and II, and C5–C6 or C6–C7 for III; *c*, ω-chain: C13–C20 for 8-*epi*-PGF$_{2\alpha}$, C11–C18 for I and II, and C9–C16 for III; *d*, C15–C20 for 8-*epi*-PGF$_{2\alpha}$, C13–C18 for I and II, and C11–C16 for III.

Ion assignment	Loss of	*m/z*			
		8-*epi*-PGF$_{2\alpha}$	Metabolite I	Metabolite II	Metabolite III
M^+		584	556	558	530
$[M - 15]^+$	$^{\cdot}CH_3$	569	541	543	515
$[M - 31]^+$	$^{\cdot}OCH_3$				499
$[M - 71]^+$	$^{\cdot}CH_2-(CH_2)_3-CH_3$	513	485	487	
$[M - 90]^+$	TMSOH	494	466	468	440
$[M - 15 - 90)]^+$	$^{\cdot}CH_3$ + TMSOH	479	451	453	425
$[M - 31 - 90)]^+$	$^{\cdot}OCH_3$ + TMSOH			437	409
$[M - 71 - 90]^+$	$^{\cdot}CH_2-(CH_2)_3-CH_3$ + TMSOH	423	395	397	369
$[M - 90 \times 2]^+$	TMSOH × 2	404	376	378	350
$[M - 90 - a]^+$	TMSOH + *a*	353	353	353	
$[M - 71 - 90 \times 2]^+$	$^{\cdot}CH_2-(CH_2)_3-CH_3$ + TMSOH × 2	333[a]	305[a]	307[a]	279[a]
$[M - 71 - 90 - 116]^+$	TMSOH + $^{\cdot}CH_2-(CH_2)_3-CH_3$ + *b*	307	279	281	253
$[TMSO-CH=CH-CH=OTMS]^+$		217	217	217	217
c^+		199	199	199	199
$[TMSO=CH-OTMS]^+$		191	191	191	191
d^+		173	173	173	173

[a] base peak.

its ME-TMS derivative (Fig. 2*A*) showing the ions expected in agreement with the fragmentation pattern described in Table II. Another abundant β-oxidation metabolite was identified in this preparation as 2,3,4,5-tetranor-8-*epi*-PGF$_{2\alpha}$ on the basis of the EI mass spectrum and GC behavior (*C* value: 20.12). Its mass spectrum (Fig. 2*B*) was almost identical to that reported by Green (8) for the corresponding metabolite of PGF$_{2\alpha}$. 2,3,4,5-Tetranor-8-*epi*-PGF$_{2\alpha}$ could be observed in the C18 SPE but not in the IAC extract. This result indicates that immunosorbent C does not extract this metabolite, and therefore we cannot presently exclude nor prove its presence in urine. 2,3-Dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ was a minor product in the hepatocyte preparation, as judged by NICI-MS analysis of PFB-TMS derivatives of C18 and IAC extracts. In the hepatocyte preparation incubated with PGF$_{2\alpha}$, we confirmed the identity of 2,3-dinor-5,6-dihydro-PGF$_{2\alpha}$ and 2,3,4,5-tetranor-PGF$_{2\alpha}$ but could not find 2,3-dinor-PGF$_{2\alpha}$, either in SPE or in IAC extracts examined by both EI- and NICI-MS, similar to what had been observed in rat urine. The same products of PGF$_{2\alpha}$ metabolism were identified by Sago *et al.* (21) in rat hepatocytes. The EI mass spectra of PGF$_{2\alpha}$ metabolites (not shown) were very similar to those published previously (8) and to those we have shown here for the corresponding metabolites of 8-*epi*-PGF$_{2\alpha}$. This similarity was expected, because it had been observed for the parent epimeric compounds, 8-*epi*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ (not shown).

Urinary Metabolite I versus Hepatocyte-derived 2,3-Dinor-8-epi-PGF$_{2\alpha}$—The immunoaffinity behavior and GC-MS characteristics of urinary Metabolite I *versus* hepatocyte-derived 2,3-dinor-8-*epi*-PGF$_{2\alpha}$, taken as a reference, were compared directly as follows. Hepatocyte medium and human urine were immunoextracted in parallel on two distinct antibodies (immunosorbents B and C) and derivatized to PFB-TMS, PFB-tBDMS, and PFB-BB-TMS. Samples were then analyzed by GC-NICI-MS in the SIR mode in the conditions described above, but with a lower GC temperature programming rate (10 °C/min), resulting in retention times of 14.45, 18.02, and 16.32 min for the PFB-TMS (*m/z* 541), PFB-tBDMS (*m/z* 667), and PFB-BB-TMS (*m/z* 463) derivatives of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$. In all these combinations of immunoselective capture, derivative formation, GC separation and mass-selective detection, Metabolite I behaved identically to 2,3-dinor-8-*epi*-PGF$_{2\alpha}$. The retention times of the three derivatives of Metabolite I were 14.45, 18.02, and 16.32 min. When similar amounts of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ and Metabolite I were co-injected, the peak area increased proportionally, whereas the width at half-maximum remained unchanged.

Selectivity of Immunoextraction—The three anti-8-*epi*-PGF$_{2\alpha}$ immunosorbents tested in this study selectively capture their nominal antigenic ligand from a complex matrix such as urine (16). As shown above, only immunosorbent B and C cross-reacted with other endogenous PGF$_2$ compounds. We tested the stereoselectivity of immunosorbents B and C against five available Class IV F$_2$-isoprostane diastereoisomers, namely 15*R*-8-*epi*-PGF$_{2\alpha}$, 15*S*-12-*epi*-PGF$_{2\alpha}$, 15*R*-12-*epi*-PGF$_{2\alpha}$, 15*S*-*ent*-12-*epi*-PGF$_{2\alpha}$, and 15*R*-*ent*-12-*epi*-PGF$_{2\alpha}$. Together with 8-*epi*-PGF$_{2\alpha}$, these compounds comprise almost the complete array (six of eight) of the Class IV F$_2$-isoprostanes predicted as most abundant, *i.e.* those bearing the alkyl chains in the *cis* position. Neither immunosorbent captured any of the compounds tested (<1% recovery). In addition, immunosorbent C, which efficiently captures 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$, cannot recognize its enantiomer, *ent*-2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ (<1% recovery).

Endogenous Levels in Human Urine—Endogenous 8-*epi*-PGF$_{2\alpha}$ and its metabolites were excreted in comparable amounts in human urine (Fig. 3). In smokers, who excreted higher amounts of 8-*epi*-PGF$_{2\alpha}$ in agreement with previous observations (16, 22), 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ increased in parallel with the parent compound (Fig. 3). Urinary excretion rate of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ was highly correlated to that of 8-*epi*-PGF$_{2\alpha}$ in a group of five smokers and five nonsmokers ($r = 0.89$, $p = 0.0005$). A weaker but significant correlation was observed for 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ ($r = 0.77$, $p = 0.01$).

Naproxen given to healthy nonsmokers ($n = 4$) did not alter the urinary excretion of the three compounds (pre- *versus* post-treatment values: 8-*epi*-PGF$_{2\alpha}$, 8.14 ± 2.05 *versus* 8.63 ± 1.51 ng/h; 2,3-dinor-8-*epi*-PGF$_{2\alpha}$, 8.95 ± 2.30 *versus* 9.51 ± 0.39 ng/h; 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$, 6.37 ± 1.30 *versus* 7.11 ± 1.04 ng/h).

DISCUSSION

Free radical-mediated nonenzymatic peroxidation of membrane-bound arachidonic acid results in the formation of F$_2$-isoprostanes, a complex family of 64 compounds isomeric to PGF$_{2\alpha}$ (1, 2). These products are found both esterified to tissue and plasma lipids and in the free form in body fluids, so that

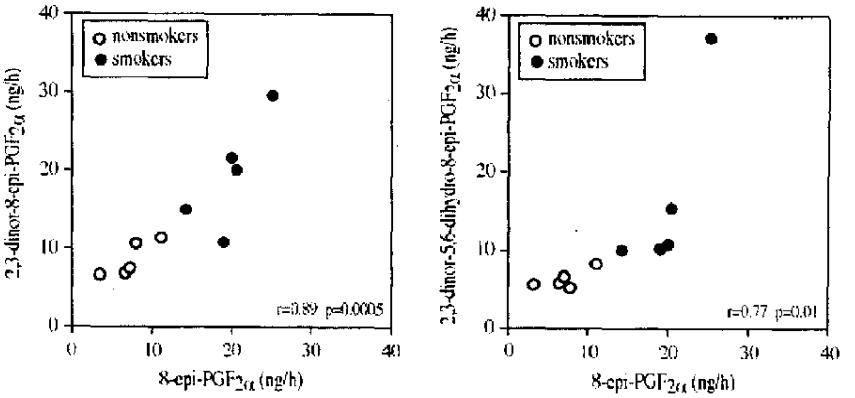

FIG. 3. **Correlation between urinary excretion of 8-*epi*-PGF$_{2\alpha}$ and its metabolites in a group of healthy volunteers (five nonsmokers and five smokers).**

they can be used to evaluate local or systemic lipid peroxidation *in vivo* (2, 3). In humans, increased F_2-isoprostane levels have been found in different physiopathological conditions putatively associated with free radical-mediated oxidant damage, such as atherothrombotic disease, diabetes, and cigarette smoking (2–4).

Assay of urinary F_2-isoprostanes and in particular of distinct major isomers such as 8-*epi*-PGF$_{2\alpha}$ and isoprostane F$_{2\alpha}$-I is of special interest for human studies, because the collection is noninvasive, the measurement is time-integrated, and the analytes are stable and cannot be formed artifactually *ex vivo* as in plasma (2–4). Although 8-*epi*-PGF$_{2\alpha}$ may also be formed enzymatically by cyclooxygenase (3), it has been repeatedly shown that its urinary excretion mainly reflects its free radical-mediated formation, at least in humans (3, 23). To evaluate 8-*epi*-PGF$_{2\alpha}$ overall formation *in vivo*, it would be useful to identify and measure its major urinary metabolites. In fact, considering that catabolism of 8-*epi*-PGF$_{2\alpha}$ may be variable in different subjects or under particular physiopathological conditions, the additional assay of degradation products would improve the significance and accuracy of these measurements. Identification of endogenous metabolites would also be useful to define new F_2-isoprostane analytical targets for assaying lipid-rich body fluids without the risk of overestimates due to autooxidation artifacts *ex vivo* (2, 4).

To date, a single major metabolite of 8-*epi*-PGF$_{2\alpha}$, *i.e.* 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$, has been identified in urine by Roberts *et al.* (10). In that study, tracer amounts of [^{3}H]8-*epi*-PGF$_{2\alpha}$ were given to a volunteer whose urine was then mixed with the urine of a monkey treated with 500 μg of 8-*epi*-PGF$_{2\alpha}$. The major radioactive human metabolites could thus be followed during urine work-up, with enough co-migrating unlabeled products from the monkey to allow identification by EI-GC-MS.

Using a different approach aimed at revealing endogenous metabolites normally present in urine, we have now shown that at least two major metabolites of 8-*epi*-PGF$_{2\alpha}$ are excreted at a rate similar to that of the parent compound. In addition to confirming the presence of 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$, we have in fact found another β-oxidation product of similar abundance excreted under basal conditions in humans. The same products were found in rat urine.

A possible explanation for 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ escaping detection in the study of Roberts *et al.* (10) might lie in an incomplete chromatographic separation from its Δ^5 saturated analogue. In fact, we observed that although 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ were easily separable by GC as PFB-TMS derivatives, they coeluted when analyzed in similar conditions as ME-TMS derivatives. Assuming that (i) in the experiment of Roberts *et al.* (10), the two compounds had coeluted both during radioactive high pressure liquid chromatography and during GC analysis of ME-TMS derivatives and (ii) urinary 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$ had been much more abundant than 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ in the treated monkey, the latter metabolite might have gone undetected in the pooled human/monkey urine sample assayed by GC-MS.

The collective evidence that Metabolite I found in urine is the 2,3-dinor metabolite of 8-*epi*-PGF$_{2\alpha}$ can be summarized as follows. As deducted from EI and NICI mass spectral data, Metabolite I has the structure of a 7,9,13-trihydroxy-dinorprost-3,11-dienoic acid. Therefore it might be any endogenous 2,3-dinor-PGF$_2$ compound, excluding the cyclooxygenase-derived 2,3-dinor-PGF$_{2\alpha}$, which elutes separately in our GC conditions. Metabolite I cannot therefore derive from a F_2-isoprostane other than Class IV, *i.e.* those with a 9,11,15-trihydroxy-prost-5,13-dienoic acid structure. Its stereochemical structure is most likely that of 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ because (*a*) the 2,3-dinor metabolite formed by hepatocytes from authentic 8-*epi*-PGF$_{2\alpha}$ behaved identically to urinary Metabolite I when analyzed by GC-NICI-MS as PFB-TMS, PFB-tBDMS, or PFB-BB-TMS and by GC-EI-MS as ME-TMS; (*b*) the two distinct anti-8-*epi*-PGF$_{2\alpha}$ immunosorbents (B and C) used to extract both urinary Metabolite I and hepatocyte-derived 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ do not cross-react with several Class IV F_2-isoprostane diastereoisomers; and (*c*) immunosorbent C displays enantioselectivity toward 2,3-dinor-5,6-dihydro-8-*epi*-PGF$_{2\alpha}$. Finally, Metabolite I increases in rat urine after administration of 8-*epi*-PGF$_{2\alpha}$.

We have identified another β-oxidation product of 8-*epi*-PGF$_{2\alpha}$, 2,3,4,5-tetranor-8-*epi*-PGF$_{2\alpha}$, in a preparation of isolated rat hepatocytes incubated with the authentic compound, but at this time we could not test for its presence in urine, because none of our available anti-8-*epi*-PGF$_{2\alpha}$ immunosorbents displayed cross-reactivity with this metabolite. The experiment with isolated rat hepatocytes comparing metabolism of 8-*epi*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ in identical conditions has preliminarily revealed some stereoselectivity in the β-oxidation pathway, as may be expected for compounds that are epimers at the α-chain-bearing carbon. In fact, although a tetranor metabolite (C16:1) was found for both of 8-*epi*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$, their respective major C18 metabolites were 2,3-dinor-8-*epi*-PGF$_{2\alpha}$ and 2,3-dinor-5,6-dihydro-PGF$_{2\alpha}$.

Profile measurements of 8-*epi*-PGF$_{2\alpha}$ and its metabolites have allowed us to note some interindividual variation in metabolism, which would imply that overall formation of 8-*epi*-PGF$_{2\alpha}$ might be estimated more accurately with the additional monitoring of the metabolites, but this aspect has to be specifically addressed with a broader sample. In smokers, the two metabolites increase in parallel with the parent compound,

although correlation with 2,3-dinor-8-*epi*-$PGF_{2\alpha}$ was stronger than that of 2,3-dinor-5,6-dihydro-8-*epi*-$PGF_{2\alpha}$. Additional observations will be needed to determine whether smokers excrete significantly different fractions of the two metabolites. In any case, their correlation with 8-*epi*-$PGF_{2\alpha}$ not only confirms their identity as degradation products but also indirectly strengthen the hypothesis that urinary 8-*epi*-$PGF_{2\alpha}$ mainly reflects the systemic rather than renal formation of the compound (23).

Treatment of volunteers with a dose of naproxen significantly inhibiting cyclooxygenase activity *in vivo* (23) did not reduce excretion of either 8-*epi*-$PGF_{2\alpha}$, as we have observed previously (23), or its metabolites. In contrast, we observed the expected reduction of $PGF_{2\alpha}$ and its assigned metabolites (data not shown). These results further confirm the identity of the various immunoextracted metabolites belonging to the cyclooxygenase-dependent pathway ($PGF_{2\alpha}$ series) *versus* those deriving from free radical-mediated lipid peroxidation (8-*epi*-$PGF_{2\alpha}$ series). A collateral finding in this study is the identification of endogenous 2,3-dinor-$PGF_{2\alpha}$ in human urine, an intermediate product that has not been detected earlier as a metabolite of exogenous $PGF_{2\alpha}$ (6, 7).

In conclusion, we have identified and measured two major endogenous degradation products of 8-*epi*-$PGF_{2\alpha}$ in human and rat urine. These metabolites may be quantified in addition to their parent compound to better assess 8-*epi*-$PGF_{2\alpha}$ formation *in vivo*. This study has also shown the usefulness of exploiting cross-reactivity of immobilized antibodies for capturing endogenous unknowns for structural analysis by GC-MS.

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